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(57) Abstract

A method for stimulating bone formation in an animal includes the step of administering to the animal an effective amount of a conjugate of a growth factor and a hydrophilic polymer. Also, a composition for treating osteopenic bone disease includes a conjugate of a growth factor and a hydrophilic polymer. Preferred conjugates include, for example, a TGF-β as the growth factor, and a polyethylene glycol as the hydrophilic polymer. Hydrophilic polymer-conjugated growth factors according to the invention can stimulate bone formation at lower dose levels at which the growth factor, unmodified, is ineffective; and hydrophilic polymer-conjugated growth factors according to the invention promote a net increase in bone formation at higher dose levels at which the growth factor, unmodified, causes a net reduction in bone mass, owing to stimulation by the growth factor of bone resorption together with bone formation. An inhibitor of bone resorption is optionally added to decrease bone resorption and thus enhance bone formation.

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CONJUGATES OF GROWTH FACTOR AND BONE RESORPTION INHIBITOR

Cross-Reference to Related Applications

This is a continuation-in-part of pending United States Patent Application Serial No. 08/051,508, filed April 22, 1993.

Background of the Invention

Technical Field

This invention relates to the use of transforming growth factors in treatment of systemic disease, and, particularly, to the use of molecules in any of the "TGF-β Superfamily" of proteins, such as the beta-type transforming growth factors ("TGF-βs"), the bone morphogenetic proteins ("BMPs"), the activins, and the inhibins, for promoting bone formation *in vivo*, particularly for treatment of osteopenic bone diseases.

Background Art

Various of the so-called growth factors can be administered systemically for therapeutic effect. Many such growth factors produce harmful or undesirable systemic effects when administered in quantities sufficient to produce the desired therapeutic effect. It is generally recognized that harmful or undesirable effects of therapeutic agents can be reduced by targeted delivery of the agent, by which a relatively smaller quantity of the agent administered to the subject localizes at the targeted

site in a relatively higher concentration. In one approach to targeted delivery of an agent, a targeting molecule having an affinity for the targeted tissue, such as a monoclonal antibody that binds cell surface receptors of targeted cell types, is conjugated to a molecule of the agent. Administered systemically, the antibody-agent conjugate circulates until it reaches a cell of the target type, whereupon the antibody binds the cell surface receptor, localizing the antibody-agent at the targeted site.

The family of peptides known as TGF-β can regulate both cell growth and cell differentiation. Depending upon the particular cell type, the peptides of the TGF-β family can stimulate or inhibit cell proliferation. Almost all tissues from all species of animals which have been examined contain TGF-βs of some type.

Copending U.S. Patent Application Serial No. 07/698,467 describes compositions for treating bone loss that include a bone growth factor, such as TGF-β, activin, or bone morphogenic protein ("BMP"), chemically conjugated (preferably via a crosslinker) to a targeting molecule having an affinity for bone, such as tetracycline, calcein, bisphosphonate, polyaspartic acid, polyglutamic acid, aminophosphosugars, or estrogen. Preferred crosslinkers, according to the '467 application, include PEG having average molecular weights between about 200 and about 10,000 daltons.

Several examples of references in the art to modifying proteins by conjugation to polymers, to alter the solubility, antigenicity, and physiological clearance of the protein may be cited. U.S. Patent No. 4,179,337 describes coupling polypeptides such as enzymes and insulin to polyethylene glycol ("PEG") or polypropylene glycol ("PPG") of 500 to 20,000 daltons molecular weight to provide physiologically active

water soluble compositions having reduced immunogenicity; U.S. Patent No. 4,261,973 describes reducing the immunogenicity of several proteins by conjugating the proteins with polyethylene glycol ("PEG") or polypropylene glycol ("PPG"). U.S. Patent No. 4,301,144 describes increasing the oxygen carrying capacity of hemoglobin by conjugating the hemoglobin with PEG and other polymers. EP 0 98,110 describes increasing the half-life of an enzyme or an interferon by coupling with a polyoxyethylene-polyoxypropylene ("POE-POP") block polymer. Abuchowski *et al.* (1984), *Cancer Biochem. Biophys.*, 7:175-86, describes reduced immunogenicity and increased half-lives in serum of a variety of enzymes conjugated with PEG. Davis *et al.* (1981), *Lancet*, 2:281-83, describes modifying the enzyme uricase by conjugating with PEG to provide uric acid metabolism in serum having a long halflife and low immunogenicity; Nishida *et al.* (1984), *J. Pharm. Pharmacol.*, 36:354-55, describes orally administering PEG-uricase conjugates to chickens, demonstrating decreased serum uric acid. Inada *et al.* (1984), *Biochem. & Biophys. Res. Comm.*, 122:845-50, describes rendering lipoprotein lipase soluble in organic solvents by conjugation with PEG; Takahashi *et al.* (1984), *Biochem. & Biophys. Res. Comm.*, 121:261-65, describes conjugating horseradish peroxidase (HRP) with PEG to render the enzyme soluble in benzene. Abuchovski *et al.* (1977), *J. Biol. Chem.*, 252(11):3578 ff., describes conjugating bovine serum albumin with PEG resulting in reduced immunogenicity and extended circulating life in the blood; and Abuchovski *et al.* (1977), *J. Biol. Chem.*, 252(11):3582 ff. describes conjugating bovine liver catalase with PEG resulting in reduced immunogenicity and enhanced half lives in the blood, and substantially retained enzymatic activity.

Summary of the Invention

We have discovered that a conjugate of a recombinant TGF-β and a hydrophilic polymer such as a polyethylene glycol ("PEG"), administered to an animal *in vivo*, can be substantially more effective for stimulating bone formation than unmodified TGF-β; and, in particular, a PEG-TGF-β conjugate can be effective for stimulating bone formation *in vivo* when administered at dose levels at which unmodified TGF-β alone is ineffective. Moreover, substantially less bone resorption results from PEG-TGF-β conjugate administration *in vivo* than from administration of unmodified TGF-β at dose levels at which unmodified TGF-β is effective in stimulating bone formation *in vivo*.

The present invention also offers *in vivo* combination therapy for stimulating new bone formation through the combined administration of the PEG-TGF-β and an agent which inhibits bone resorption.

Disclosure of Invention

In one aspect, in general, the invention features a method for treating a systemic disease condition by administering to the animal an effective amount of a conjugate of a growth factor and a hydrophilic polymer. In particular embodiments, the growth factor is a bone growth factor and the systemic disease condition is treated by stimulating bone formation.

A "bone growth factor", as that term is used herein, includes any of the TGF-β family of growth factors, and includes activin and bone morphogenetic proteins ("BMP").

In preferred embodiments the bone growth factor is a TGF-β, such as TGF-β2, and more preferably is a recombinant TGF-β, such as recombinant TGF-β2. "TGF-β", as that term is used herein, includes TGF-β1,

TGF-β2, TGF-β3, TGF-β4 and TGF-β5, and heterodimers thereof; and, more broadly, "TGF-β" means and includes any molecule that competes with binding of the native form of TGF-β for any of the cell surface TGF-β-binding proteins, including any of the TGF-β receptors types I through IX that have to date been characterized.

A "hydrophilic polymer", as that term is used herein, is a synthetic or natural polymer having an average molecular weight and composition that render the polymer essentially water soluble. Most hydrophilic polymers have this property by virtue of their having a sufficient number of oxygen atoms (less frequently nitrogen atoms) available for forming hydrogen bonds in aqueous solution. Hydrophilic polymers generally used herein include PPG, PEG, POE, polytrimethylene glycols, polylactic acid, and derivatives thereof. Particularly suitable hydrophilic polymers include a polyethylene glycol $-(CH_2CH_2O)_n-$ ("PEG"), a polypropylene glycol $-(CH_2CH_2CH_2O)_n-$ ("PPG"), or a hydrophilic carbohydrate or polysaccharide; preferred hydrophilic polymers include, for example, PEGs or PPGs having a molecular weight between 200 and 100,000, such as, for example, PEG 5000, PEG 1700, or PEG 35,000. Preferably, where the hydrophilic polymer is a PEG and the growth factor is a TGF-β2, 1 - 14 molecules of the hydrophilic polymer, and more preferably 1 - 7 molecules of the hydrophilic polymer, are attached to each TGF-β2 homodimer; that is, the PEG is present with TGF-β2 homodimer in a molar ratio preferably in the range about 1:1 to about 14:1, and more preferably in the range about 1:1 to about 7:1. Other suitable polymers include POE-POP block polymers and copolymers.

As used herein, a "conjugate" of a hydrophilic polymer and a growth factor is a

composition in which the hydrophilic polymer is attached to the growth factor *via* a covalent bond.

As used herein, the term "inhibition of bone resorption" refers to prevention of bone loss, especially the inhibition of removal of existing bone either from the mineral phase and/or the organic matrix phase, through direct or indirect inhibition of osteoclast formation or activity. Thus, inhibitor of bone resorption refers to agents that prevent bone loss by the direct or indirect inhibition of osteoclast formation or activity.

In another general aspect, the invention features a composition for stimulating bone deposition, including a conjugate of a growth factor and a hydrophilic polymer.

In still another aspect, the invention features a composition for stimulating bone deposition, including a conjugate of a growth factor and a hydrophilic polymer and an inhibitor of bone resorption.

While we do not wish to be bound to any particular theory of operation of the hydrophilic polymer — growth factor conjugates according to the invention, we offer the following observations.

The growth factor may be brought into more effective proximity to the tissue site on which the growth factor is effective, when conjugated with a hydrophilic polymer according to the invention. This may result from a more or less specific affinity of the hydrophilic polymer (or of the conjugate) for the tissue. PEG-TGF-β2 conjugates according to the invention may effectively localize to bone following systemic administration. As a result, the action of TGF-β2 on both osteoblasts and osteoclasts may be enhanced or prolonged; and an *in vivo* inhibition of osteoclast activity by TGF-β2 (as can be observed *in vitro*) can be effected, resulting in a net increase in

bone. For stimulation of bone formation, therefore, preferred hydrophilic polymers may (either in and of themselves or when conjugated with a bone growth factor) have a specific affinity for bone tissue.

Alternatively, the pharmacokinetics of the growth factor may be altered by conjugation with a hydrophilic polymer according to the invention. For example, in the bone formation stimulation example, a differential effect of the bone growth factor (such as TGF-β2) on osteoblasts (as compared with osteoclasts) may result from conjugation with the hydrophilic polymer (such as PEG), resulting in a net increase in bone. Osteoblasts and osteoclasts may have different receptors for TGF-β, for example, binding different portions of a particular TGF-β molecule or binding with a greater or lesser affinity; and such differences may result in a differential effect of the hydrophilic polymer-conjugated growth factor on these two cell types.

We have further discovered that reduced toxic effects result from administration of a conjugate of a higher molecular weight PEG (for example, PEG 35,000) and a TGF-β2 at lower molar ratios, or lower degrees of PEG conjugation, that is fewer moles of PEG covalently attached to TGF-β (for example, in the range about 1:1 to about 3:1), at TGF-β2 dose levels effective for stimulating bone formation.

In another general aspect, therefore, the invention features a method for stimulating bone formation in an animal by administering to the animal a PEG-TGF-β2 conjugate in which the PEG has a molecular weight between about 5 kd and about 100 kd, and preferably about 35 kd, and in which the higher molecular weight PEG is present with the TGF-β2 homodimer in a molar ratio in the range about 1:1 to about 3:1.

As will be appreciated, the preferred molar ratio for a given combination of hydrophilic polymer and growth factor depends among other variables upon the molecular size of the hydrophilic polymer. In general, lower molar ratios of a larger than of a smaller hydrophilic polymer can be effective in a conjugate with a given growth factor. The mechanism of systemic clearance is different for circulating molecules of different sizes. The clearance mechanism for a particular growth factor-hydrophilic polymer conjugate depends among other factors upon the molecular size of the whole conjugate (*see, e.g.*, M.J. Knauf *et al.* (1988), *J. Biol. Chem.*, 263(28): 15064-70???. TGF-βs, for example, have a molecular size (dimer) about 26,000; preferred conjugates of hydrophilic polymers with TGF-βs have a molecular size at least about 55,000 or 60,000; such molecular sizes can be obtained by conjugation of PEG 5000 and a TGF-β at molar ratios at least about 6:1, or by conjugation of PEG 35,000 and a TGF-β at a molar ratio of at least about 1:1. Apparently, as the examples below demonstrate, a lower molar ratio of higher molecular size PEGs in PEG-TGF-β conjugates can be more effective than a higher molar ratio of lower molecular size PEGs. For conjugated TGF-β2, for example, the activity is significantly reduced or lost at molar ratios of PEG 5000 to TGF-β2 greater than 7:1.

The hydrophilic polymer — growth factor conjugate can be administered in conjunction with a bone resorption inhibitor. In preferred embodiments the conjugate is administered in conjunction with a bone resorption inhibitor such as, for example, an estrogen, a bisphosphonate or a calcitonin. Administration of the conjugate can commence either prior to, at the same time as, or following administration of the bone resorption inhibitor. The conjugate and bone resorption inhibitor can be

administered at least partly concurrently. Where administration is to be simultaneous, the conjugate and the bone resorption inhibitor may or may not be combined in a single composition.

Drugs which prevent bone loss and/or add back lost bone are often first tested in the ovariectomized rat. This animal model is well established in the art (see, for example, Wronski, et al. (1985) Calcif. Tissue Int. 37:324-328; Kimmel, et al. (1990) Calcif. Tissue Int. 46:101-110; and Durbridge, et al. (1990) Calcif. Tissue Int. 47:383-387; these references are hereby incorporated in their entirety). Wronski, et al. ((1985) Calcif. Tissue Int. 43:179-183)) describe the association of bone loss and bone turnover in the ovariectomized rat.

Examples of inhibitors of bone resorption include estrogens such as estradiol, tamoxifen, bisphosphonates, calcitonins, or other small peptides or molecules that may inhibit bone resorption. (Turner, et al. (1987) J. Bone Mineral Res. 2:115-122; Wronski, et al. (1988) Endocrinology 128:681-686; and Wronski, et al. (1989) Endocrinology 125:810-816; Pfeilshifter, et al. (1987) Proc. Natl. Acad. Sci. U.S.A. 84:2024-2028; Turner, et al. (1988) Endocrinology 122:1146-1150). An example of a small peptide is echistatin, which includes the arginine-glycine-aspartate (RGD) sequence which is recognized by some cell surface adhesion receptors and apparently disrupts osteoclast interactions (Fisher et al. (1993) Endocrinology 132: 1411-13). Another example of a bone resorption factor is OPF or osteoclastpoietic factor (PCT Publication WO 93/01827 published 4 February 1993).

The entire molecule of a particular inhibitor may be used, or alternatively, only a functional part of the inhibitor molecule may be used.

Bisphosphonates include, but are not limited to, pamidronate, alendronate, residronate and tiludronate.

Description of Preferred Embodiments

There follow illustrative protocols for making hydrophilic polymer — growth factor conjugates according to the invention, showing by way of example protocols for production of PEG-TGF-β2 conjugates. Other hydrophilic polymers than polyethylene glycols, and other growth factors than TGF-βs can be coupled to make the conjugates of the invention. Adjustment of steps and particular parameters given in the protocols can be made as appropriate for the particular materials and according to the custom of the particular laboratory, all without undue experimentation and within the ordinary skill of the art.

Examples of chemistries for conjugating or coupling proteins to hydrophilic polymers have been summarized in the art. *See, e.g.*, Tyle *et al.*, Eds. (1990), *Targeted Therapeutic Systems*, Marcel Dekker, NY; and Means *et al.* (1990), *Bioconjugate Chemistry*, Vol. 1, pp. 2-12.

Modes of Carrying out the Invention

Construction of hydrophilic polymer — growth factor conjugates

Generally, the growth factor can be conjugated at a reactive group to the selected hydrophilic polymer or polymers. Reactive groups on the growth factor include, but are not limited to, carboxyl groups of the polypeptide C-terminus or of aspartic acid or glutamic acid residues, amino groups of the polypeptide N-terminus or of lysine residues, imidazole functions of histidine residues and phenolic functions of tyrosine residues, sulfhydryl groups of residues, and guanidine groups of arginine residues.

The molar ratio of hydrophilic polymer molecules to growth factor molecules can be controlled by selection of specific conjugation chemistries (for example, by reacting the polymer predominantly with primary amine substituents on lysine residues, of which there are a fixed number on any given growth factor molecule) and, as will be appreciated, by control of reaction conditions such as temperature or pH.

For example, the bone growth factor TGF-β contains a number of available amino, carboxyl, and hydroxy groups that may be used to bind the hydrophilic polymer. The hydrophilic polymer may be connected using a "linking group", as the native hydroxy or amino groups in TGF-β and in the hydrophilic polymer frequently require activation before they can be linked. Thus, for example, a compound such as a dicarboxylic anhydride (*e.g.*, glutaric or succinic anhydride) can be employed to form a polymer derivative (polymer glutarate or polymer succinate, *e.g.*, using PEG and succinic anhydride, a PEG-succinate), which can then be activated by esterification with a convenient leaving group (*e.g.*, N-hydroxysuccinimide, N,N'-disuccinimidyl oxalate, N,N'-disuccinimidyl carbonate, or the like). The activated polymer is then allowed to react with the bone growth factor, to form the hydrophilic polymer — bone growth factor conjugate.

Preferred dicarboxylic anhydrides for use in forming polymer-glutarate compositions include glutaric anhydride, adipic anhydride, 1,8-naphthalene dicarboxylic anhydride, and 1,4,5,8-naphthalenetetracarboxylic dianhydride. Suitable crosslinkers have chemistries well known in the art, and they are commercially available.

The resulting hydrophilic polymer — growth factor conjugate can be purified using a standard

technique, such as by reverse-phase high performance liquid chromatography ("RP-HPLC"), size-exclusion HPLC ("SEC-HPLC"; *e.g.*, tetrahydrogel-HPLC), or ion-exchange chromatography. RP-HPLC is preferably performed using a C18 column using gradient elution with 80 - 100% acetonitrile, ethanol or isopropanol with 0.1% trifluoroacetic acid ("TFA") as the eluting solvent. For SEC-HPLC, a preferred running buffer can be 5 mM sodium acetate at pH 5.5, preferably including an organic solvent, urea or a detergent.

PEG—TGF-β Conjugates

For example, a PEG—TGF-β conjugate can be made according to the following protocols, which make specific reference to formation of rTGF-β2(PEG 5000)$_6$ from PEG 5000 and rTGF-β2 and to formation of rTGF-β2(PEG 35,000)$_{1-3}$ from PEG 35,000 and rTGF-β2.

TGF-β is difficult to dissolve in solutions of appropriate pH for coupling to hydrophilic polymers. In a preferred protocol, therefore, the TGF-β is lyophilized in the absence of a carrier protein or is held in solution in acid/organic solvent. For use TGF-β is dissolved in a mild acid, preferably about 10 mM HCl, in the presence of a disaggregating reagent such as an organic solvent (*e.g.*, 40% acetonitrile, ethanol or propylene glycol; or a combination of organic solvents such as 30% ethanol, 15% propylene glycol). The solution is then neutralized by adding a base, preferably NaOH (1 N solution) in buffered saline (*e.g.*, phosphate buffered saline). The final solution preferably contains about 40 - 50% DMSO or CH_3CN to solubilize the TGF-β and to prevent aggregation, thus preserving TGF-β activity.

rhTGF-β2(PEG 5000)$_6$

In a preferred protocol, the activated PEG 5000 is a succinimydyl ester made, for example, as follows. Monomethylpolyethylene glycol, average

molecular weight about 5,000 daltons, ("mPEG 5000") is reacted with glutaric anhydride to form mPEG glutarate. The glutarate derivative is then reacted with N-hydroxysuccinimide to form a succinimydyl mPEG glutarate. The succinimydyl ester is then capable of reacting with free amino groups (lysine residues) on the TGF-β to form a TGF-β — PEG conjugate.

The following specific protocol was used for making rhTGF-β2(PEG 5000)$_6$ in a 5 mg batch from recombinant human TGF-β2 (rhTGF-β2) and methoxypolyethylene glycol succinimydyl succinate 5000 (M-S-PEG 5000). 5.2 mg/ml rhTGF-β2 (200 nmol rhTGF-β2) available from Genzyme Corp., Cambridge, MA, was combined with 5.5 ml acetonitrile, 1.5 ml HPLC-grade water, 1.4 ml 10x phosphate buffered saline (10x PBS) and 100 μl 0.1 N NaOH to make about 13.70 ml total volume, with a protein concentration of 0.38 mg/ml, 48% organic solvent, at pH 7.2. M-S-PEG 5000 (Sigma, lot 11H8040) (8 μmol; 40 mg), stored dry at -20 °C, was added (either directly or dissolved in acetonitrile) at a molar ratio of 40:1, to the rhTGF-β2 mixture, and permitted to react for 2 hours at room temperature. The resulting mixture was then diluted at least 1:3 with 0.1% TFA, the pH was adjusted to between pH 2 and pH 4 using 1% TFA, and the mixture was fractionated by C18-RP-HPLC (Vydac 218TP510, 1 x 25 cm). Solvent A was 0.1% TFA and solvent B was 90% acetonitrile in A. In a 1%/min. gradient of A to B, unreacted TGF-β2 eluted first at 41% solvent B followed by TGF-β with one PEG 5000 molecule attached. In a subsequent gradient at 0.5%/min. from 41-50% solvent B, TGF-β2 having increasingly higher amounts of PEG 500 eluted from the column. By stepping up solvent B to 62%, a PEG-TGF-β2 conjugate having a high molar ratio of PEG 5000 to TGF-β2 eluted. Fractions were further analyzed by SDS-PAGE under reduced and unreduced conditions.

TGF-β2 fractions having more than three PEG 5000 molecules attached were pooled for evaluation.

rhTGF-β2(PEG 35,000)$_{1-3}$

The following specific protocol was used for making rhTGF-β2(PEG 35,000)$_{1-3}$ from recombinant human TGF-β2 (rhTGF-β2) and bis-polyethylene glycol succinimydyl carbonate 35,000 (BSC-PEG 35,000). 2.6 mg/ml rhTGF-β2 in HCl, 20% EtOH, made according to the above-referenced production protocol, was combined with 2.0 ml acetonitrile, 1.0 ml propylene glycol (PG), 1.0 ml HPLC-grade water, 1.0 ml 10x PBS and 100 µl 0.1 N NaOH to make about 8.7 ml total volume. BSC-PEG 35,000, provided by Milton Harris, University of Alabama, Huntsville, AL, was added either (A) in a 5:1 mol ratio (20 mg) or (B) in a 2:1 mol ratio (8 mg), to the rhTGF-β2 mixture, to yield a protein concentration 0.268 mg/ml, 36.3% organic solvent, 10.3% PG, at pH 7.2. The mixture of rhTGF-β2 and BSC-PEG 35,000 was permitted to react for 90 min. at room temperature, pH 7.2. The resulting mixture was then diluted at least 1:3 with 0.1% TFA, the pH was adjusted to between pH 2 and pH 4 using 1% TFA, and the TGF-β2(PEG 35,000) was purified by C18-RP-HPLC according to the purification protocol referenced above. The resulting HPLC fractions were analyzed under non-reduced and reduced conditions on SDS-PAGE, 5-15% gradient. The protein concentration of pooled purified samples was determined at OD 280 nm, and aliquots were prepared for use as described above.

Pharmaceutical compositions containing hydrophilic polymer–growth factor conjugate

The PEG-TGF-β compositions of the invention are preferably administered by parenteral routes, intravenous injection, intranasal or bronchial aerosol, and the like. The compositions can be employed in sustained release vehicles, such as from a

slow-release carrier, or from a sustained release device that may be surgically implanted subdermally or within the peritoneal cavity.

Pharmaceutical formulations for administration of the hydrophilic polymer — bone growth factor according to the invention generally include an osteogenically effective amount of the bone growth factor in addition to a pharmaceutically acceptable excipient. Suitable excipients include most carriers approved for parenteral administration, including water, saline, Ringer's solution, Hank's solution, as well as solutions of lactose, dextrose, ethanol, glycerol, albumin, and the like. The compositions may optionally include stabilizers, antioxidants, antimicrobials, preservatives, buffering agents, surfactants, and other accessory additives, such as propylene glycol. A preferred mode of administration includes about 10-50% propylene glycol. The more preferred mode includes about 40% propylene glycol.

By way of example, saline or phosphate-buffered saline (PBS) can be a preferred vehicle for parenteral administration of rTGF-β—PEG. (Martin, *Remington's Pharmaceutical Sciences*, Mack Publ. Co., current edition, includes a discussion of suitable vehicles for parenteral administration; sections thereof relating to excipient vehicles and formulating are hereby incorporated by reference.)

The hydrophilic polymer — growth factor compositions of the invention may be formulated as solutions or suspensions, or they may be lyophilized for later reconstitution.

An "osteogenically effective amount" of hydrophilic polymer — growth factor conjugate, that is, an amount sufficient to effect treatment in the subject, will depend upon the particular growth factor and the particular hydrophilic polymer used in the

conjugate and the number of polymer molecules attached to each growth factor molecule in the conjugate, as well as the nature and severity of the condition to be treated, the age and general health of the subject, the specific activity of the composition, and other factors that may be determined by the practitioner of ordinary skill in the art of treating bone disease. Generally, doses of conjugate in the range 0.001 to 10 μg/kg body weight, and more preferably in the range 0.001 to 1 μg/kg body weight, and most preferably in the range 0.01 to 0.1 μg/kg body weight, should be effective. Hereinafter, weights or volumes given /kg refer to /kg of body weight.

Because the hydrophilic polymer — bone growth factor conjugates of the invention are substantially more effective for stimulating bone formation *in vivo* than the bone growth factor alone, effective systemic dosages of the conjugate according to the invention are much lower and may be less frequently administered than are effective dosages of the corresponding unconjugated growth factor. For stimulation of bone growth using PEG-TGF-β2 conjugates, for example, the number of molecules of the conjugate that need be administered systemically to achieve the desired treatment effect can be at least 10- to 100-fold lower than the number of molecules of unmodified TGF-β2 that must be administered to achieve the desired treatment effect. Thus, conjugate compositions according to the invention that have relatively lower activities as determined by an *in vitro* assay can be effective for treatment *in vivo*.

An effective dose for estrogen is about 1 μg/kg to about 1 mg/kg of body weight. An effective dose for bisphosphonates is quite variable but generally between about 0.05 μg/kg to about 15 mg/kg of body weight. An effective dose for calcitonin is

about 0.05 IU (International Units or Medical Research Council Units)/kg to about 2.5 IU/kg of body weight.

The combination of PEG-TGF-β and an inhibitor of bone resorption is useful for treating bone fractures, defects, and disorders which result in weakened bones such as osteoporosis (including postmenopausal, age-related and idiopathic), osteoarthritis, Paget's disease, osteohalisteresis, osteomalacia, bone loss resulting from multiple myeloma and other forms of cancer, and bone loss resulting from side effects of other medical treatment (such as steroids).

Industrial Applicability

The hydrophilic polymer — growth factor conjugates according to the invention can be administered for treatment of diseases where bone loss occurs, such as, for example, osteoporosis.

There follow examples illustrating effects on bone metabolism of administration of the hydrophilic polymer — bone growth factor conjugates of the invention, employing an animal model, as well as combination therapy of PEG-TGF-β with inhibitors or bone resorption.

Comparison of effects of rTGF-β2(PEG 5000)$_6$ and rTGF-β2 on bone formation in mice

In one example, groups of mice were treated by subcutaneous injection with two different doses of recombinant TGF-β2 ("rTGF-β2"), with two different doses of polyethylene glycol 5000 conjugated (6:1) with recombinant TGF-β2, or "rTGF-β2(PEG 5000)$_6$", or with a vehicle control. A variety of analyses were used to determine the effects of the treatments on bone formation.

More particularly, 10-week-old male C3H mice were divided into five treatment groups of six mice each. Mice in the first group were treated with mouse

serum albumin (MSA), a vehicle control; those in the second and third groups were treated with rTGF-β2 at doses of 20 μg and 0.1 μg per mouse (760 μg and 3.8 μg per kg body weight), respectively; and those in the fourth and fifth groups were treated with rTGF-β2(PEG 5000)$_6$ at doses of 0.1 μg and 0.02 μg per mouse (3.8 μg and 0.76 μg per kg body weight), respectively. (The body weight index is 26.4 g, the average weight of the animals at the start of the study.) Treatments were performed daily for eleven days by subcutaneous injections of 100 μl each in the tailbase. Demeclocycline was used as a fluorochrome label on day 1, and calcein was used on days 6 and 10 for histomorphometric analyses.

A variety of analyses were performed. Body weights of the animals were measured daily. At the end of the treatment period the animals were sacrificed, and analyses were performed as follows: hematology (white cell counts, red cell counts, packed cell volume (PCV), hemoglobin, platelet counts, differential blood cell counts); thymic cellularity; histology (liver, kidney, spleen); and bone histomorphometry (femur epiphysis).

The results can be summarized as follows. Mice treated with rTGF-β2(PEG 5000)$_6$ in daily doses of 3.8 μg/kg body weight showed increases in indices of bone formation in all measured parameters, with close to normal values for indices of bone resorption, resulting in an overall increase in cancellous bone mass (increases in percent trabecular area, trabecular thickness, trabecular number, decreases in trabecular separation). In contrast, bone formation in mice treated with unmodified rTGF-β in the same daily doses of 3.8 μg/kg body weight was not significantly different from bone formation in controls. Both bone formation and bone resorption indices were stimulated in mice treated with the higher dose of rTGF-β2, which

resulted in a net reduction of trabecular bone mass (decreases in percent trabecular area, trabecular thickness, trabecular number; increases in trabecular separation).

Comparison of effects of rTGF-β2(PEG 35,000)$_{1-3}$, rTGF-β2(PEG 5000)$_6$, and rTGF-β2 on bone

In another example, mice were treated by subcutaneous injection with rTGF-β2 (two different doses), with rTGF-β2(PEG 5000)$_8$ or rTGF-β2(PEG 5000)$_6$ or rTGF-β2(PEG 5000)$_4$ (two different doses for each molar ratio), with rTGF-β2(PEG 35,000)$_{1-3}$ (two different doses), or with a vehicle control. A variety of analyses were used to determine the effects of the treatments on bone cell morphology.

More particularly, 8 week-old-male C3H mice were divided into treatment groups. Mice in the first group were treated with MSA-PEG 5000, as a control; those in the second group were treated with 3 μg r-TGF-β2 admixed with PEG 5000 (uncomplexed); those in the third and fourth groups were treated with rTGF-β2 at doses of 15 μg and 3 μg per mouse, respectively; those in the fifth, sixth and seventh groups were treated with 3 μg per mouse rTGF-β2(PEG 5000) at molar ratios (PEG: rTGF-β2) of 8:1, 6:1, and 4:1, respectively; those in the ninth, tenth and eleventh groups were treated with 0.6 μg per mouse rTGF-β2(PEG 5000) at molar ratios 8:1, 6:1, and 4:1, respectively; and those in the eighth and twelfth groups were treated with rTGF-β2(PEG 35,000)$_{1-3}$ at doses of 3 μg and 0.6 μg per mouse, respectively. Treatments were performed once daily for four days by subcutaneous injections of 100 μl each in the tailbase.

A variety of analyses were performed. Body weights of the animals were measured daily. At the

end of the treatment period the animals were sacrificed, and analyses were performed as follows: hematology (white cell counts, red cell counts, packed cell volume (PCV), hemoglobin, platelet counts, differential blood cell counts); thymic cellularity; and qualitative femur histology.

The results can be summarized as follows. Very highly significant proliferation of osteoblast-like cells was observed in the femur slides of mice treated at 3 μg with rTGF-β2(PEG 5000)$_6$ or rTGF-β2(PEG 5000)$_4$ or rTGF-β2(PEG 35,000)$_{1-3}$, as compared with controls. Such cell proliferation was interpreted as bone stimulation. Less dramatic but still highly significant proliferation of osteoblast-like cells was observed in femur slides obtained from mice treated at 0.6 μg with rTGF-β2(PEG 5000)$_6$ or rTGF-β2(PEG 5000)$_4$ or rTGF-β2(PEG 35,000)$_{1-3}$. Significant proliferation of osteoblast-like cells was observed on the cortical and trabecular bone surfaces of the femur obtained from rTGF-β2(PEG 35,000)$_{1-3}$ treated mice. Generally, each dosage showed slightly greater effects on bone tissue than rTGF-β2(PEG 5000) at molar ratios 6:1 or 4:1.

Significant increases in RBC, hemoglobin and PCV, and significant decreases in platelets and thymic cellularity appeared in mice treated at 3 μg with rTGF-β2(PEG 5000)$_6$ or rTGF-β2(PEG 5000)$_4$ or rTGF-β2(PEG 35,000)$_{1-3}$; significant decreases in bone marrow (femur) cellularity appeared in mice treated at 3 μg with rTGF-β2(PEG 5000)$_6$ or rTGF-β2(PEG 5000)$_4$. Weight loss was high (about 20%) in mice treated at 3 μg with rTGF-β2(PEG 5000)$_6$ or rTGF-β2(PEG 5000)$_4$ or rTGF-β2(PEG 35,000)$_{1-3}$.

Mice treated with rTGF-β2(PEG 5000)$_8$ gained weight slightly over the study period and showed no significant changes in hematologic, bone marrow or thymic analysis.

At the lower dosage (0.6 μg), while mice treated with rTGF-β2(PEG 5000)$_6$ showed significant increases in RBC, hemoglobin and % PCV, mice treated at 0.6 μg with rTGF-β2(PEG 5000)$_4$ or rTGF-β2(PEG 35,000)$_{1-3}$ showed no significant changes in hematologic or bone marrow or thymus analysis. Moreover, while weight loss was very high (about 9%) in mice treated with rTGF-β2(PEG 5000)$_6$ even at the lower dose, weight loss was only moderate (about 3.3%) in mice treated at 0.6 μg with rTGF-β2(PEG 5000)$_4$; and mice treated at 0.6 μg with rTGF-β2(PEG 35,000)$_{1-3}$ lost less than 1% of body weight over the study period—and this treatment was about as effective in stimulating proliferation of osteoblast-like cells (which was interpreted as bone growth stimulation) as were the higher dosages of rTGF-β2(PEG 5000) at molar ratios 6:1 and 4:1.

Body weight losses, reduction in thymus cellularity and hematological changes are all toxic effects resulting from PEG-TGF-β treatment. In summary, coupling TGF-β with higher molecular size PEGs (for example, in the range about 10,000 to 100,000) at lower molar ratios (for example, in the range about 1:1 to 3:1) can produce PEG-TGF-β conjugates having greater effectiveness in bone stimulation at similar dosages and having similar effectiveness at lower dosages with substantially reduced toxic effects, than coupling TGF-β with lower molecular size PEGs (for example, in the range about 200 to 10,000) at higher molar ratios (for example, as high as about 8 PEG:1 TGF-β).

Comparison of effects of PEG-rTGF-β2 with and without bone resorption inhibitors on bone formation in rats

In one example, groups of ovariectomized (OVX) rats were treated by subcutaneous injections of PEG-rTGF-β2 with and without the bone resorption

inhibitor estradiol as a preliminary examination largely of safety.

More particularly, female rats were ovariectomized at 12 weeks of age and then lost bone until the start of the study when the rats were 95 weeks old. The OVX rats were given a dietary intake restricted to minimize the body weight gain that normally follows ovariectomy.

During the day prior to the study, OVX rats had their spinal bone mineral density (BMD) measured by DEXA. Based on spine BMD values, the OVX rats were then randomly assigned among the study groups described below, with reasonable attempts made to have mean BMD values similar for all groups. The rats were given subcutaneous injections (0.1 ml/rat) for 8 weeks (days 1-56) of vehicle, estradiol (10 μg/kg) and/or PEG-rTGF-β2 (1 μg/kg). PEG-rTGF-β2 was given every day for 3 weeks, then 3 times a week for 4 weeks, and then every day during the last week. The PEG-rTGF-β2 vehicle was 40% propylene glycol with <2% ethanol in PBS with pH adjusted to 7.2. The study groups were organized as follows:

Group	Injection(s)
sham	PEG-rTGF-β2 vehicle
OVX	None - sacrificed at the start of the study
OVX	PEG-rTGF-β2 vehicle
OVX	PEG-TGF-β2 vehicle + estradiol (10 μg/kg)
OVX	PEG-rTGF-β2 (1 μg/kg)
OVX	PEG-rTGF-β2 (1 μg/kg)+ estradiol (10 μg/kg)

All values below show means ± standard error of the mean, with number of rats in parentheses.

Table 1

Initial Body Weight (g) and Spine BMD (mg/cm^2) Values

Treatment	Body Weight	Spine BMD
Sham	411±9(4)	252±5(4)
Pretreatment OVX	361±6(7)	204±6(7)
OVX + Vehicle	371±8(10)	202±4(10)
Estradiol	376±12(7)	204±5(7)
PEG-rTGF-β2	363±7(10)	204±16(6)
PEG-rTGF-β2 + Estradiol	363±10(11)	198±13(7)

For each rat, the individual body weights for days 21, 42 and 56 were subtracted from the day 0 value to obtain the individual changes and are shown in Table 2 below.

Table 2

Body Weight Changes (grams)
during the Course of the Study

Treatment	3 Weeks	6 Weeks	8 Weeks
Sham	-19±7(4)	-12±6(4)	-32±12(4)
OVX + Vehicle	-4±3(10)	-2±5(10)	-5±4(10)
Estradiol	-12±3(7)	-25±7(7)	-28±6(6)
PEG-rTGF-β2	-6±5(10)	-3±7(10)	-11±10(10)
PEG-rTGF-β2 + Estradiol	-17±4(11)	-18±7(11)	-27±7(11)

All rats were scanned at the beginning and at 3, 6, and 9 weeks into the study. For each rat the difference between their individual spine BMD values (mg/cm^2) at 3, 6 and 9 weeks and the initial values measured on day 0 value were calculated to obtain the individual changes in spine BMD reported in Table 3.

Table 3

Spine BMD Changes during the Course of the Study

Treatment	3 Weeks	6 Weeks	8 Weeks
Sham	5±9(4)	2±6(4)	2±9(4)
OVX + Vehicle	5±5(10)	1±4(10)	2±5(10)
Estradiol	6±4(7)	5±5(7)	6±4(6)
PEG-rTGF-β2	-16±9(6)	-11±7(6)	-18±7(6)
PEG-rTGF-β2 + Estradiol	3±3(7)	3±5(7)	5±4(7)

After sacrifice at 8 weeks, the tibia were removed and scanned. The tibia lengths and projected areas are presented in Table 4. The tibia bone mineral content, bone mineral density and bone mineral apparent density data are given in Table 5. The bone mineral densities for tibial cortical bone, metaphysis and epiphysis are given in Table 6.

Table 4

Tibia Lengths and Projected Areas

Treatment	N	Length(mm)	Area (cm^2)
Sham	4	42.8±0.4	1.45±0.05
Pretreatment OVX	7	43.0±0.5	1.49±0.03
OVX + Vehicle	10	42.4±0.4	1.45±0.02
Estradiol	7	42.3±0.2	1.44±0.01
PEG-rTGF-β2	10	42.0±0.3	1.41±0.02
PEG-rTGF-β2 + Estradiol	11	42.1±0.3	1.45±0.03

Table 5

Tibia Global BMC, BMD and BMAD Values

Treatment	N	BMC (mg)	BMD (mg/cm^2)	BMAD (mg/cm^3)
Sham	4	335±25	230±12	191±9
Pretreatment OVX	7	311±11	209±4	171±3
OVX + Vehicle	10	303±7	209±2	174±1
Estradiol	7	312±9	217±5	180±4
PEG-rTGF-β2	10	293±12	207±6	174±4
PEG-rTGF-β2 + Estradiol	11	303±13	208±6	173±4

Table 6

Tibia Cortical Bone, Metaphysis and Epiphysis BMD Values

Treatment	N	Cortical Bone	Metaphysis (mg/cm^2)	Epiphysis (mg/cm^3)
Sham	4	238±8	217±27	266±25
Pretreatment OVX	7	204±4	209±3	209±6
OVX + Vehicle	10	203±3	205±3	210±4
Estradiol	7	208±6	219±7	226±8
PEG-rTGF-β2	10	199±6	204±7	214±9
PEG-rTGF-β2 + Estradiol	11	199±6	209±7	215±9

Table 7 indicates that the estradiol dose was sufficient to normalize the uterine size, but PEG-rTGF-β2 had little if any effect.

Table 7

Uterine Weight (g) at Sacrifice

Treatment	N	Uterine Weight
Sham	4	0.85±0.11
OVX + Vehicle	10	0.16±0.02
Estradiol	6	0.80±0.13
PEG-rTGF-β2	10	0.25±0.08
PEG-rTGF-β2 + Estradiol	10	0.60±0.05

It appeared that on sacrifice there was less cutaneous fibrosis with PEG-rTGF-β2 than with TGF-β2. Less fibrosis at the injection site is an important clinical advantage, because fibrosis can interfere with absorption and cause unsightly scar-like tissue.

Dose Effect of Recombinant TGF-β2-$(PEG\ 5000)_6$ on Bone Remodeling in Bilaterally Ovariectomized Adult Rats

Recruitment and increased activity of osteoblast cell population was observed in previous experiments on normal mice which had been treated with subcutaneous administration of PEG-TGF-β2. In the eleven-day mouse study, all measured bone formation parameters were increased; whereas, bone resorption parameters remained unchanged. The net result was an increase in trabecular bone mass in the group of animals treated with 0.4μg/kg body weight of rTGF-β2-$(PEG\ 5000)_6$ injected subcutaneously over an 11-day period.

This purpose of this study is to:

a) test the ability of rTGF-β2$(PEG\ 5000)_6$ to promote bone formation in osteopenic rat skeleton after bilateral ovariectomy;

b) compare effects on bone of three different doses of PEG-TGF-β2 when injected daily (versus the effect of the same dose injected on a twice-a-week basis); and

c) compare dose response of PEG-TGF-β2 administration on bone-forming and bone-resorbing cells over the two- and four-week periods.

One hundred and thirty five female, Sprague-Dawley rats are obtained at 90 days of age and housed for one week to acclimate. After acclimation, 23 randomly chosen animals are sham-operated; whereas, the remaining 112 females are bilaterally ovariectomized by the dorsal approach under ketamine/xylazine anesthesia. Eight weeks post sham surgery or ovariectomy, animals are randomly divided in eight experimental groups and are treated as shown:

Group	Treatment	Frequency of treatment	Number of Animals Euthanized at Each Time Point		
			day 0	day 14	day 28
1. Sham	vehicle	daily	5	9	9
2. OVX	vehicle	daily	5	9	9
3. OVX	5μg/kg	daily	0	7	7
4. OVX	1μg/kg	daily	0	7	7
5. OVX	0.2μg/kg	daily	0	7	7
6. OVX	5μg/kg	2/week	0	7	7
7. OVX	1μg/kg	2/week	0	7	7
8. OVX	0.2μg/kg	2/week	0	7	7

Five animals from groups 1 and 2 serve as baseline controls and show bone status in sham and OVX groups before treatment with PEG-TGF-β2.

Seven to nine animals from each group are sacrificed 14 and 28 days after treatment started. All animals receive intraperitoneal injections of fluorescent bone markers: calcein (10 mg/kg) is given at day 0 and day 11 to animals euthanized at day 14 and at day 14 and day 21 to animals sacrificed at day 28. Declomycin (25 mg/kg) is administered at day 7 to animals scheduled to be sacrificed on day 14 and on

day 21 to animals scheduled to be sacrificed on day 28.

Body weights are monitored for all animals weekly. Animals are necropsied by exsanguination from the vena cava under deep anesthesia with ketamine/xylazine.

Livers from the Group 3 mice killed on day 28 are processed for histopathology.

Both femurs, tibias, lumbar vertebral bodies, and mandibles are collected, fixed in ethanol and processed for various histomorphometric analyses. Distal femoral metaphyses from right femurs are embedded undecalcified for static and dynamic histomorphometry of cancellous bone. Also, cortical bone histomorphometry is performed on cross-sections from right tibias at the tibio-fibular junction. The proximal portion of the same tibias is demineralized, embedded in paraffin, cut in thin longitudinal sections (4-5μm). These sections are used for osteoid and cellular measurements (osteoid surface, osteoid maturation time, osteoblast surface, osteoclast surface, osteoclast number, and number of nuclei per osteoclast). Bodies of lumbar vertebrae (L5 and L6) are embedded decalcified and serve for histomorphometric measurements of cancellous bone.

Left tibias which have been cleaned of soft tissues are used for determination of wet, dry and ash weight. Also, if necessary, calcium and phosphorus are determined from ash. Left femurs and mandibles are stored undecalcified at -70°C for eventual additional measurements.

Other Embodiments

Other embodiments are within the following claims.

Claims

1. A method for stimulating bone formation in an animal, comprising the step of administering to the animal an effective amount of a conjugate of a growth factor and a hydrophilic polymer.

2. The method of claim 1 wherein said growth factor is a TGF-β.

3. The method of claim 2 wherein said growth factor is a recombinant TGF-β.

4. The method of claim 2 wherein said growth factor is a TGF-β2.

5. The method of claim 2 wherein said growth factor is a recombinant TGF-β2.

6. The method of claim 1 wherein said hydrophilic polymer has an affinity for bone *in vivo*.

7. The method of claim 1 wherein said hydrophilic polymer comprises a polyethylene glycol.

8. The method of claim 7 wherein said polyethylene glycol has a molecular weight in the range of about 200 daltons to about 100,000 daltons.

9. The method of claim 8 wherein said polyethylene glycol has a molecular weight of about 5000 daltons.

10. The method of claim 7 wherein said polyethylene glycol has a molecular weight of about 35,000 daltons.

11. The method of claim 1 wherein said polyethylene glycol has a molecular weight of about 55,000 daltons to about 130,000 daltons.

12. The method of claim 1 wherein said hydrophilic polymer comprises a polypropylene glycol.

13. The method of claim 1 wherein the hydrophilic polymer and the growth factor in said conjugate are in a molar ratio in the range of about 1:1 to about 14:1.

14. The method of claim 13 wherein the hydrophilic polymer and the growth factor in said conjugate are in a molar ratio in the range of about 1:1 to about 7:1.

15. The method of claim 10 wherein the growth factor is a TGF-β and wherein the polyethylene glycol and the TGF-β in said conjugate are in a molar ratio in the range of about 4:1 to about 7:1.

16. The method of claim 11 wherein the growth factor is a TGF-β and wherein the polyethylene glycol and the TGF-β in said conjugate are in a molar ratio in the range of about 1:1 to about 4:1.

17. The method of claim 1, further comprising administering to the animal a bone resorption inhibitor.

18. The method of claim 17 wherein said bone resorption inhibitor comprises an estrogen.

19. The method of claim 17 wherein said bone resorption inhibitor comprises a bisphosphonate.

20. The method of claim 17 wherein said bone resorption inhibitor comprises a calcitonin.

21. The method of claim 17 wherein said growth factor — hydrophilic polymer conjugate is administered at least partially concurrently.

22. A composition for promoting bone formation *in vivo*, comprising a conjugate of a growth factor and a hydrophilic polymer.

23. The composition of claim 22 wherein said hydrophilic polymer has an affinity for bone *in vivo*.

24. The composition of claim 22 wherein said growth factor is a TGF-β.

25. The composition of claim 24 wherein said growth factor is a recombinant TGF-β.

26. The composition of claim 24 wherein said growth factor is dimer containing a TGF-β2 subunit.

27. The composition of claim 25 wherein said growth factor is a recombinant TGF-β2.

28. The composition of claim 27 wherein said growth factor is a recombinant human TGF-β2.

29. The composition of claim 19 wherein said hydrophilic polymer comprises a polyethylene glycol.

30. The composition of claim 29 wherein said polypropylene glycol has a molecular weight between about 200 daltons and about 100,000 daltons.

31. The composition of claim 30 wherein said polyethylene glycol has a molecular weight of about 5000 daltons.

32. The composition of claim 30 wherein said polyethylene glycol has a molecular weight of about 1700 daltons.

33. The composition of claim 30 wherein said polyethylene glycol has a molecular weight of about 35,000 daltons.

34. The composition of claim 22 wherein the growth factor is a TGF-β and wherein the polyethylene glycol and the TGF-β in said conjugate are in a molar ratio in the range of about 1:1 to about 4:1.

35. The composition of claim 22, further comprising a bone resorption inhibitor.

36. The composition of claim 35 wherein said bone resorption inhibitor comprises an estrogen.

37. The composition of claim 35 wherein said bone resorption inhibitor comprises a bisphosphonate.

38. The composition of claim 35 wherein said bone resorption inhibitor comprises a calcitonin.

INTERNATIONAL SEARCH REPORT

Inte ional application No.
PCT/US94/01662

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/36, 37/43
US CL :514/8,12,21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/8,12,21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS; DIALOG: MEDLINE; WPI
SEARCH TERMS: TGF-BETA, BONE RESORPTION, BONE GROWTH, HYDROPHILIC POLYMER

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	US, A, 5,208,219 (OGAWA ET AL) 04 MAY 1993, COLUMNS 5-6; COL. 7, LINES 36-41; COL. 8, LINES 64-68.	1-38
Y	US, A, 5,118,667 (ADAMS ET AL) 02 JUNE 1992, COL. 3, LINES 27-35.	1-38
Y	US, A, 4,179,337 (DAVIS ET AL) 18 DECEMBER 1979, COL. 2, LINES 53-58.	1-38
Y	US, A, 5,162,430 (RHEE ET AL) 10 NOVEMBER 1992, COL. 5.	1-38

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

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